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The characterization of defense responses to fungal infection in alfalfa

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Abstract. The enzyme activity and transcript level of three enzymes involved in flavonoid biosynthesis, phenylalanine ammonia-lyase (PAL), cinnamic acid 4-hydroxylase (CA4H), and isoflavone reductase (IFR), were monitored in alfalfa seedlings (*Medicago sativa*) that had been challenged with the fungal pathogen *Colletotrichum trifolii*. In many legumes, a pathogen–host infection leads to an induced synthesis of fungitoxic phytoalexins produced via these key enzymes. For example, when alfalfa is exposed to an avirulent fungal type, phytoalexins are produced by the plant providing protection from additional exposure to a virulent fungal race. This defensive plant protection response was accompanied by increases in transcript levels of PAL, CA4H and IFR gene expression, by increases in PAL enzyme activity, and by production of the end product phytoalexins, medicarpin and sativan. The expression of these defense genes and PAL enzyme activity were significantly greater in seedlings responding to combined inoculations of avirulent and virulent fungi, compared to plants inoculated with the avirulent fungus alone. Inoculation with the virulent race alone elevated the gene transcripts compared with control plants but these levels were less than found in plants inoculated with the avirulent race or those inoculated with both. Production of gene transcripts reached its peak in treated plants after 49 h. The phytoalexins medicarpin and sativan increased rapidly and reached maximal levels 97 h after inoculation with the avirulent fungal race. The plants challenged with combined inoculations of the avirulent and the virulent fungi showed significantly greater accumulation of medicarpin than in any other treatment. These results suggest that the increased medicarpin accumulation produced in induced resistant tissues, following challenge inoculation with virulent race, is attributable to increased expression of genes of flavonoid biosynthesis.

Key words: alfalfa, anthracnose, inducible defense responses, leguminosae, lucerne, plant/fungal interactions

Introduction

Anthracnose, caused by *Colletotrichum trifolii* Bain & Essary, is a major fungal disease of alfalfa (*Medicago sativa* L.), reducing growth and

foliage yield (Barnes et al., 1969; Elgin et al., 1981). Four physiological races of the fungus have been identified (Welty and Mueller, 1979; Elgin and Ostazeski, 1982; O'Neill et al., 1997; Mackie et al., 2003). Resistance to race 1 and race 2 is inherited in alfalfa as two independent, dominant loci, named An_1 and An_2, respectively (Elgin and Ostazeski, 1985; Elgin and O'Neill, 1988). Resistance in alfalfa appears to result from a rapid induction of the early enzymes of phenylpropanoid biosynthesis, with correspondingly early accumulation of isoflavonoids medicarpin and sativan (Dewick and Martin, 1979; Ingham, 1979; Paiva et al., 1994; Edwards et al., 1995; Sallaud et al., 1997). The biosynthetic pathway leading to these phytoalexins is expressed *de novo* by fungal infection or other eliciting conditions (Smith et al., 1971; Paiva et al., 1994).

One potential IPM control method for anthracnose in alfalfa might be the biological control strategy of induced resistance. Enhancement of resistance is a common response of plants and is accomplished by the inoculation of a plant with an inducer agent and second challenge inoculation with a pathogen. In previous reports, we showed that inoculation with avirulent *C. trifolii* race (race 1) induces resistance against disease caused by a virulent race in alfalfa (O'Neill et al., 1989). Rapid, significant increases in phytoalexin accumulation were observed in race 1 protected cotyledons (O'Neill and Saunders, 1994; O'Neill, 1996). In these tissues, even greater phytoalexin accumulation was observed immediately following challenge inoculation with race 2. The degree of disease protection from race 2 was dependent upon the spore density of race 1 inducing inoculum and was associated with significantly increased phytoalexin accumulation. These results suggested that protection may not be solely a result of defense induction in response to inoculation with an avirulent race. The induction phase may also serve to facilitate or prime, the enhanced defense expression seen upon challenge by a virulent race. There are various possible mechanisms that might explain the accumulation of flavonoids observed in induced tissues challenged by the fungi. They could be the result of *de novo* transcriptional activation of flavonoid biosynthetic pathway defense genes by race 2, or the flavonoids could be released from more complex conjugated chemical forms, or they could have arisen by other factors. The objective of the present study was to explain how changes in phytoalexins with induced plants conferred biological resistance to virulent fungal challenge.

We approached this problem by monitoring activation of key defense genes in the phenylpropanoid and isoflavonoid pathway both at the level of the mRNA and at final gene expression levels. We monitored

two enzymes in the early part of the phenylpropanoid pathway including phenylalanine ammonia-lyase (PAL) and cinnamic acid 4-hydroxylase (CA4H), the first and second key enzymes leading to many different metabolic pools including flavonoids, coumarins, and lignin-derived compounds. We also chose an enzyme late in the pterocarpan specific pathway, isoflavone reductase (IFR), which catalyzes one of the final steps in medicarpin biosynthesis (Guo and Paiva, 1995).

Materials and methods

Plant inoculations

Seeds of *Medicago sativa* cultivar Arc (resistant to race 1 and susceptible to race 2) were grown in sterilized soil, at 23 °C with a 16 h photoperiod for 14 days. Race 1 isolate 2sp2 and Race 2 isolate SB-2 of *Colletotrichum trifolii* were cultured on Potato Dextrose Agar (Sigma, St. Louis, MO) at 23 °C with a 12-h photoperiod. Spores from 7 day cultures were suspended in distilled water and filtered through cheesecloth and the spore density adjusted to 2.0×10^6 per ml with distilled water and Tween 20 (1 drop per liter of inoculum). Alfalfa seedlings were inoculated with a fungal spore sprayer containing the spore suspension at 20 psi covering all surfaces of the leaves. Following inoculation plants were incubated in a 100% humidity chamber at 23 °C for 48 h. Samples were collected for RNA and phytoalexin extraction at 4 h intervals for the first 25 h, then at 24-h intervals until 121 h after inoculation. Challenge inoculation with race 2 was performed 24 h after race 1 inoculation. Changes in relative levels of PAL, IFR, and CA4H activity or mRNA were determined in two independent experiments using different alfalfa seedlings. Duplicate samples were processed for each experiment.

RNA extraction and purification

Two grams of excised plants 1 cm above the soil surface were ground in liquid nitrogen using a mortar and pestle. Ten milliliters of Trizol reagent (Life Technologies, Inc., Gaithersburg, MD) was added to each sample and phase extracted with 2 ml of chloroform, mixed vigorously and centrifuged at $12{,}000 \times g$ for 15 min at 4 °C. RNA was precipitated from the supernatant by adding 0.7 volume of isopropanol and incubating on ice for 20 min, followed by recentrifugation at $12{,}000 \times g$ at 4 °C for 15 min. The resulting RNA pellet was washed with 75% (v/v)

ethanol, decanted, and the pellet dissolved in 0.5 ml of diethylpyrocarbonate (DEPC) treated, sterilized, deionized water. RNA levels were quantified spectrophotometrically at 260 nm.

RNA blot analysis

RNA slot blots were prepared using a Minifold II Slot Blot System (Schleicher & Schuell, Keene, NH) using standard procedures modified as described (Dyson, 1991). Each sample of 4 μg RNA was denatured with 60 μl of 20 × SSC and 40 μl of 37% (v/v) formaldehyde incubated at 65 °C for 15 min. To monitor the induced transcript levels of the gene coding for PAL, a 471 bp *Hin*dIII DNA fragment from the coding region of the alfalfa PAL gene was used as a probe (20). The expression of IFR was monitored using a 1.18 kbp *Eco*RI fragment from the coding region of the alfalfa IFR cDNA (Guo and Paiva, 1995). The probe for CA4H, was a 1.75 kbp *Bam*HI-*Sal*I fragment from the coding region described by Graham and Graham (1994). Cloned cDNA for these probes were kindly provided by Dr. Nancy Paiva, Noble Foundation, Ardmore, OK. A heterologous probe made from a cDNA clone of the 18S rRNA from soybean (Fahrendorf and Dixon, 1993) was used to ensure that equal amounts of RNA had been loaded for each sample. Labeling was performed using a random priming labeling kit (Life Technologies Inc., Gaithersburg, MD) following the manufacturer's instructions. Hybridization with ^{32}P labeled DNA probes was performed at 42 °C overnight. Pre-hybridization and hybridization buffer contained 5x Denhardt solution, 50% (v/v) formamide, 5x SSC, 10 mM EDTA, 100 μg/ml sonicated and denatured salmon sperm DNA, and 5% (w/v) SDS. The hybridized membrane was washed twice in 5x SSC and 0.1% (w/v) SDS for 15 min, twice in 1x SSC and 0.1% (w/v) SDS at 37 °C for 15 min, and finally in 0.1x SSC, 0.1% (w/v) SDS at 50 °C for 15 min. X-ray films were exposed for 3–7 days at −70 °C and developed in an automatic film processor. The mRNA levels were quantified from slot blot hybridizations using a flying-spot scanner calibrated with control samples (Shimadzu, model CS-9000U, Japan) and converted to percent of control. No observable variation of replicated samples was seen in the mRNA accumulation for multiple experiments.

Phytoalexin extraction and HPLC analysis

Approximately 1 g (FW) of seedlings were ground in liquid nitrogen with a mortar and pestle and stored at −70 °C until use. Samples that were resuspended in 4.5 ml of 80% (v/v) acetonitrile in 1 mM acetic

acid were filtered, rinsed with 0.5 ml 80% (v/v) acetonitrile, and diluted to 25% acetonitrile by addition of 1 mM acetic acid. The samples were passed through a Baker-10 SPE octadecyl (C18) disposable extraction column and washed with 2, 3 ml volumes of 25% (v/v) acetonitrile. The eluate was discarded and sample compounds adhering to the column were eluted with 3.5 ml of 100% acetonitrile evaporated under nitrogen and stored at –70 °C until analysis. Samples were resuspended in 60 μl acetonitrile, and compounds were separated by HPLC on a reverse phase C18, 4 μm column, 30 cm × 3.9 mm diameter (Waters Novapak, Waters Corporation, Milford, MA). The mobile phase was 20% (v/v) acetonitrile in 1 mM acetic acid, increasing to 60% (v/v) over 55 min, and 98% (v/v) over an additional 5 min at a flow rate was 0.8 ml per minute. A Waters 990 photodiode array UV detector monitored UV absorbance at 210–360 nm and peak analysis performed with Millennium Chromatography software (Waters Corporation, Milford, MA).

Under the extraction conditions used, isoflavonoid conjugates were hydrolyzed to their aglycones to recover total isoflavonoids. Further digestion of sample extracts was confirmed using β-glucosidase, conducted following the method of Edwards et al. (1995). HPLC analysis of enzymatically treated samples showed no differences in the chromatographic profiles.

PAL enzyme activity

After each incubation period, duplicate 1–2 g fresh weight samples of seedlings were blotted dry, weighed, and frozen at −70 °C. Samples were extracted for PAL activity as previously described (O'Neill and Saunders, 1994). PAL activity was calculated as the production of nanomoles of cinnamate per hour per milligram of protein.

Results

Alfalfa seedlings were subjected to one of four different treatments of fungal inoculation and several different biochemical and physiological parameters related to phytoalexin productions were measured for 121 h after treatment. The biochemical parameters included gene transcription, enzyme activity, and accumulation of several phytoalexins. Alfalfa seedlings inoculated with race 2 of *Colletotrichum trifolii* (compatible interaction) exhibited disease symptoms 73 h after inoculation. At 97 h, susceptible seedlings were water-soaked and at 121 h

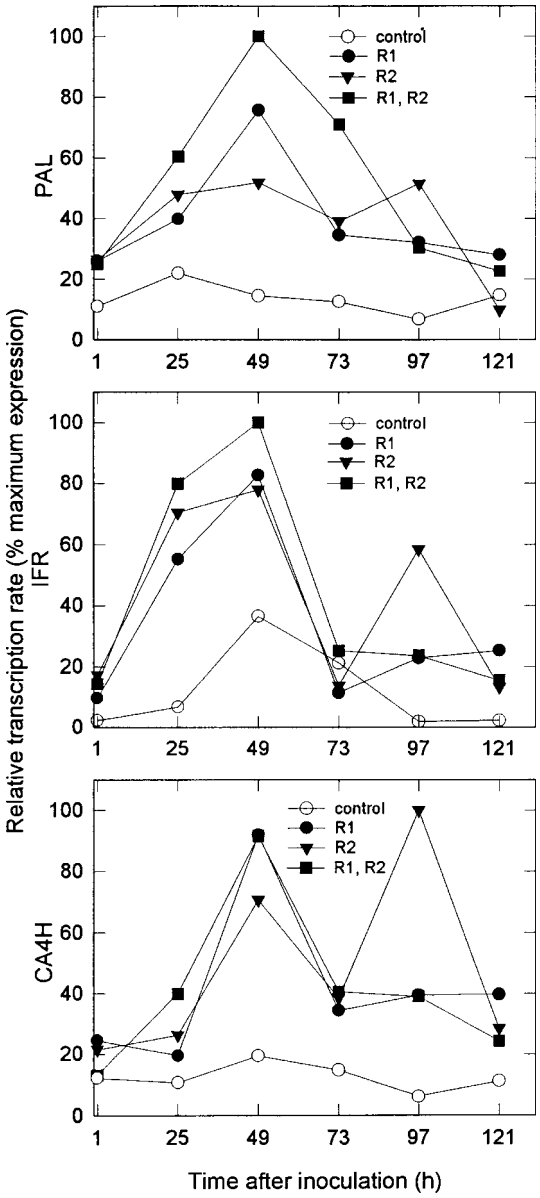

Figure 1. Transcriptional activation of PAL, IFR, and CA4H in *M. sativa* cultivar Arc following inoculations with *C. trifolii*. R1 represents the treatment in which alfalfa seedlings were inoculated with race 1 alone (incompatible interaction); R2, inoculated with race 2 alone (compatible interaction); R1, R2, initially inoculated with race 1 and challenge inoculated with race 2, 24 h later. The control represents treatment in which seedlings were sprayed with deionized water. Values are normalized to the highest value (100%) within each transcript.

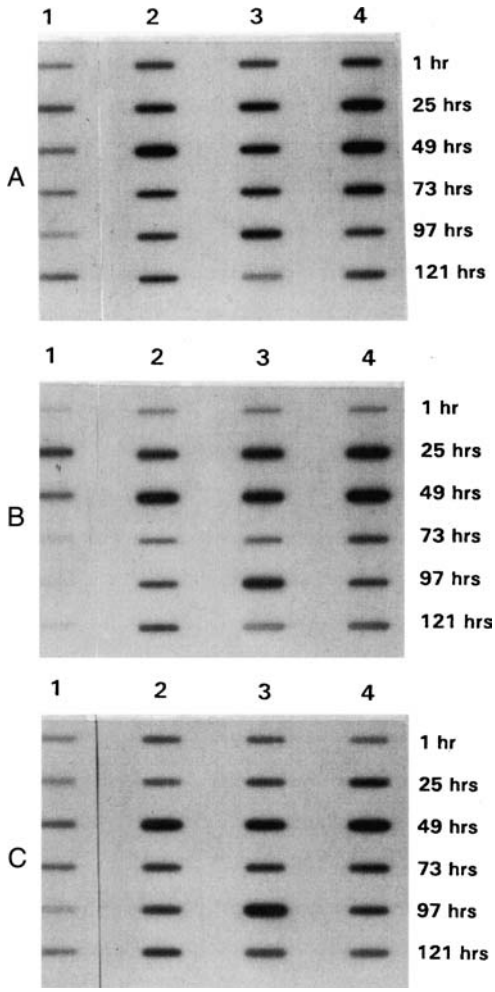

Figure 2. Autoradiographs of slot blots of treatments indicated in Figure 1. (A) Total RNA was hybridized against a 471 bp *Hin*dIII DNA fragment from the coding region of PAL; (B) Total RNA hybridized against a 1.18 kbp *Eco*RI fragment from the coding region of IFR. (C) Total RNA hybridized against a 1.75 kbp *Bam*HI-*Sal*I fragment from the coding region of CA4H. Lane 1, total RNA from plants without fungal inoculation (sprayed with deionized water only); lane 2, total RNA from plants inoculated with race 1 alone; lane 3, total RNA from plants inoculated with race 2 alone; lane 4, total RNA from plants initially inoculated with race 1 and 24 h later with race 2.

they became severely necrotic. In contrast, seedlings inoculated with race 1 or seedlings treated with race 2 following the initial race 1 inoculation remained healthy throughout the time course being measured. RNA slot-blot hybridization provides a convenient mechanism

to compare gene transcript activation because uniform hybridization areas on the membrane can be scanned by densitometry in a consistent manner between samples. These slot blots showed that fungal inoculation increased transcript levels of all the three genes studied compared with the uninoculated control plants (Figures 1 and 2). All treatments induced maximum levels of these transcripts 49 h after inoculation. PAL and IFR transcript accumulation was greater in the race 1 inoculated seedlings challenged by race 2, especially in the early stages between 25 and 49 h after inoculation (Figure 1). This pattern of enhanced response to a previous challenge was not evident with CA4H gene expression. A second increase in each of the three gene transcripts which were monitored appeared 97 h after inoculation in the race 2 susceptible lines as shown in Figure 2. It is interesting to note that the most consistent difference seen in all of the gene transcripts tested was the enhanced expression of the alfalfa inoculated with the race 2 line at the 97 h incubation point. This is correlated with the onset of the severe disease symptoms and may represent a general response of the plant to the progress of the disease. Further incubation of the race 2 treated plant resulted in a drastic decline in the physiological condition of the plant to the point where active transcription was reduced as the tissue degraded.

Figure 3 shows that medicarpin levels peaked at 97 h in those plants that had been induced with race 1 and had a subsequent race 2 challenge inoculation. Moderate levels of medicarpin were found in the seedlings inoculated with only race 1 while plants inoculated with race 2 alone and controls had minute levels of medicarpin (Figure 3). Control plants inoculated with race 1 followed by a second inoculation with race 1 again were not significantly different than a single race 1 inoculation (data not shown). The phytoalexin, sativan, showed similar patterns of accumulation peaking at 97 h in the plants inoculated either with race 1 alone or in plants treated with race 1 followed by race 2 challenge (Figure 3). Levels of sativan were very low in all other plants. Coumestrol levels, which increased to an early maximal level at 25 h in plants with race 2 inoculation, declined after longer incubations. Appreciable levels of coumestrol were detected only in plants inoculated with race 1 after 73 h of incubation (Figure 3).

PAL enzyme activity began increasing after 49 h of incubation in the race 1 inoculated plants (Figure 4). The challenge inoculation with race 2 treated plants delayed this initial increase in PAL activity over race 1 inoculations but these plants show the highest PAL activity of all treatments after 97 h of incubation.

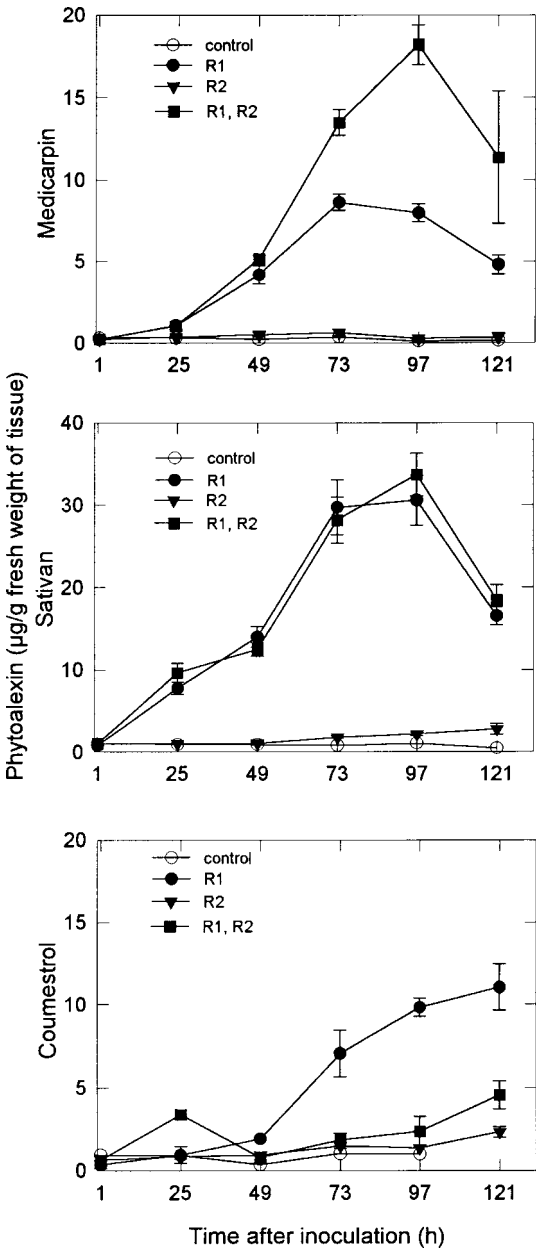

Figure 3. Accumulation of phytoalexins medicarpin, sativan, and coumestrol in seedlings of *M. sativa* cultivar Arc inoculated with *C. trifolii*. R1 represents the treatment in which alfalfa seedlings were inoculated with race 1 alone; R2, inoculated with race 2 alone; R1 R2, initially inoculated with race 1 and challenge inoculated with race 2, 24 h later. The control represents treatment in which seedlings were sprayed with deionized water only. Standard deviation of sample means are shown in error bars.

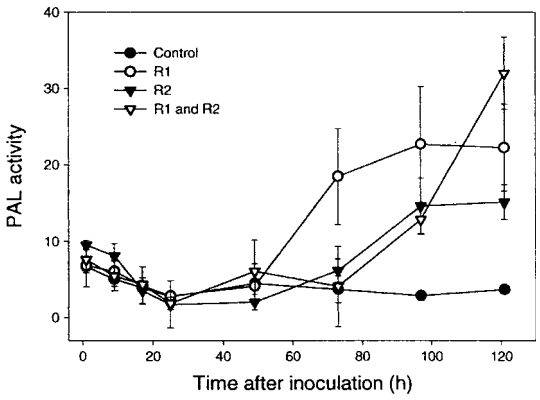

Figure 4. PAL enzyme activities of *M. sativa* inoculated with *C. trifolii*. R1 represents the treatment in which alfalfa seedlings were inoculated with race 1 alone; R2, inoculated with race 2 alone; R1, R2, initially inoculated with race 1 and challenge inoculated with race 2, 24 h later. The control represents treatments in which seedlings were sprayed with deionized water only. PAL-specific activity was calculated as the production of nanomoles of cinnamate per hour per milligram of protein. Standard deviation of sample means are shown in error bars.

Discussion

The results of this study have shown that alfalfa plants respond to an avirulent fungal inoculation by developing an induced resistance that enhances gene expression in flavonoid metabolism, increases enzymatic activity of PAL, and increases levels of medicarpin. Rapid, significant increases in the phytoalexins medicarpin, sativan, vestitol, and coumestrol were reported earlier in alfalfa cotyledons protected from anthracnose by inoculation with race 1, and challenge inoculated 24 h later with race 2 (O'Neill, 1996). The results of the present study explain that seedlings protected by race 1 and subsequently inoculated with a virulent race exhibit enhanced expression of PAL and IFR gene transcripts, coordinate with increased PAL enzyme activity and induced resistance to the virulent fungus. These patterns of gene induction and expression at the mRNA level have not been reported previously and this study has shown that they are correlated with enhanced isoflavonoid biosynthesis. Among the three genes tested, PAL activity appears to be the limiting factor in determining the presence of high levels of phytoalexins. This reflects the long-standing view that PAL is a key regulatory enzyme in the phenylpropanoid pathway (Saunders and Olechno, 1988). Based on the differences in mRNA levels produced in plants responding to fungal inoculation, PAL

activity was much larger than IFR and CA4H. Although moderate amounts of IFR and CA4H gene transcripts were present during race 2 inoculation, there was not a dramatic increase in phytoalexin accumulation. Although CA4H is known in some systems to be an inducible enzyme in response to fungal infection (Durst et al., 1996) this enzyme may also function in a constitutive manner that passes regulation of the phenylpropanoid pathway to PAL. This pathway is known to be the precursor biosynthetic pathway for many phytoalexins, flavonoids, coumarins, lignin, and most other phenolic containing plant compounds.

Accumulation of phytoalexins has been shown to be induced in a non-specific manner in suspension cell culture. Elicitor preparations from cell walls of the phytopathogenic fungus *Colletotrichum lindemuthianum* have been reported to produce high concentrations of phytoalexins in bean cells (Tepper et al., 1989) that was preceded by increased activities of isoflavonoid biosynthetic enzymes. In another study of yeast elicitor in suspension cultures of alfalfa, PAL mRNA transcripts accumulated massively within 2 h after elicitation followed by a 50-fold increase in PAL activity within 12 h (Gowri et al., 1991). Thus suspension cell cultures showed a pattern of initial increased in mRNA levels followed closely by a rise in enzyme activity and finally metabolite accumulation. Paiva et al. (1991) observed the production of medicarpin and sativan in detached alfalfa leaves during susceptible interactions with cultivar Apollo infected with *Phoma medicaginis*. The activities of two phytoalexin-specific enzymes, IFR and pterocarpan synthase (PTS), were detectable within two hours after inoculation in the *P. medicaginis* treated leaves. Increases in the amount of mRNAs for PAL, CHS, CHI and IFR were also found shortly after inoculation and enhanced levels were observed over 20 h. These results demonstrate an active and non-specific defense response in alfalfa in susceptible cultivars. It appears that increases in phytoalexin levels observed following inoculation with *C. trifolii* race 2 is also a result of non-specific elicitation, similar to that observed in elicited alfalfa suspension cells and cultivar Apollo.

There are several possible mechanisms to explain the increased levels of phytoalexins observed from induced resistant seedlings following the challenge inoculation. The increased gene expression of defense enzymes IFR and PAL from *de novo* mRNA synthesis may account for the increased levels of the medicarpin observed. It is interesting to note that not all of the phytoalexins showed the same induced resistant response as medicarpin. This would imply that there may be several mechanisms to enhance production of specific phytoalexins. While the present data

showing increases in both PAL and IFR transcription supports that contention, there are other phenolic compounds produced in alfalfa that have precursors channeled through the phenylpropanoid pathway. An alternative to de novo synthesis is the possibility that pre-formed phytoalexin conjugates in the form of isoflavone glycosides may be released upon signals from the challenge inoculation. Such conjugates have been reported in alfalfa roots, suspension cells and wounded soybean cotyledons (Tiller et al., 1994; Edwards et al., 1995). However, detectable levels of two potential conjugates, medicarpin glucoside malonate (MGM) and formononetin glucoside malonate (FGM), were not found in appreciable amounts in samples from our study (data not shown). One recent study has shown a significant correlation between isoflavonoids that are being artificially manipulated and disease resistance in alfalfa (He and Dixon, 2000).

The present study confirms the hypothesis that increased *de novo* mRNA and protein synthesis occur in induced resistant alfalfa seedlings responding to fungal inoculation by a virulent race. The inoculation of alfalfa seedlings with race 1 increased expression of some genes in flavonoid metabolism, which in turn caused rapid increases in phytoalexin synthesis. The challenge inoculation with race 2 further enhanced this metabolic response, but only on plants with the prior conditioning inoculation with race 1. While these phenomena of defense responses to multiple inoculations had been reported previously (O'Neill et al., 1989) this is the first report to provide data at the molecular level, which attributes these increases to synthesis at the gene transcript level.

Sallaud et al. (1997), suggested that the induction of genes involved in the flavonoid pathway is not necessarily sufficient to lead to the accumulation of the expected phytoalexins. They suggested that one or several post-transcriptional steps are essential in determining the nature or quantity of the flavonoids accumulated during resistance interactions. Thus the additional medicarpin accumulating in induced resistant plants challenged by a virulent race may also involve other posttranslational events that affect flavonoid production.

The phenomenon of induced and enhanced resistance offers a potential for bio-control of seedling pathogens of alfalfa. Further studies are needed to identify the component(s) from race 2 that elicit an enhanced defense response in alfalfa and result in increased medicarpin accumulation. Such elicitors may have potential for biocontrol of other foliar pathogens of alfalfa, especially if the defense response can be enhanced in a non-specific manner.

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